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Conservative substitutions in the hydrophobic core of *Rhodobacter sphaeroides* thioredoxin produce distinct functional effects

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Abstract

The internal residue Phe 25 in *Rhodobacter sphaeroides* thioredoxin was changed to five amino acids (Ala, Val, Leu, Ile, Tyr) by site-directed mutagenesis, and the mutant proteins were characterized in vitro and in vivo using the mutant *trxA* genes in an *Escherichia coli* TrxA$^-$ background. The substitution F25A severely impaired the functional properties of the enzyme. Strains expressing all other mutations can grow on methionine sulfoxide with growth efficiencies of 45–60% that of the wild type at 37°, and essentially identical at 42°. At both temperatures, however, strains harboring the substitutions F25V and F25Y had lower growth rates and formed smaller colonies. In another in vivo assay, only the wild type and the F25I substitution allowed growth of phage T3/7 at 37°, demonstrating that subtle modifications of the protein interior at position 25 (Ile/Leu or Phe/Tyr) can produce significant biological effects. All F25 mutants were good substrates for *E. coli* thioredoxin reductase. Although turnover rates and apparent K_m values were significantly lower for all mutants compared to the wild type, catalytic efficiency of thioredoxin reductase was similar for all substrates. Determination of the free energy of unfolding showed that the aliphatic substitutions (Val, Leu, Ile) significantly destabilized the protein, whereas the F25Y substitution did not affect protein stability. Thus, thermodynamic stability of *R. sphaeroides* thioredoxin variants is not correlated with the distinct functional effects observed both in vivo and in vitro.

Keywords: fluorescence; kinetic constants; protein stability; thioredoxin

Intimate packing of hydrophobic side chains at internal positions is important for protein folding and stability (Kellis et al., 1988, 1989; Lim & Sauer, 1991). Subtle disruptions of the packed protein core can result in dramatic effects on both stability and function (Dao-Pin et al., 1991; Lim et al., 1992), despite the apparent plasticity of protein backbones revealed by various structural studies (see, for example, Baldwin & Matthews, 1994). In this paper we analyze the functional effects of conservative substitutions in the hydrophobic core of *Rhodobacter sphaeroides* thioredoxin.

Thioredoxins are small (~12 kDa) heat-stable oxidoreductases implicated in many biological processes (reviewed by Holmgren [1985]). Reduced by thioredoxin reductase via NADPH, *Escherichia coli* thioredoxin serves as a hydrogen donor for enzymes such as ribonucleotide reductase and methionine sulfoxide reductase. The reduced form of the protein is an essential subunit of the T7 phage-encoded DNA polymerase and takes part in the assembly of the filamentous phages f1 and M13. The homologous enzyme from the photosynthetic bacterium *R. sphaeroides* is closely related to the *E. coli* protein (47% of amino acid sequence identities) and is able to restore the wild type phenotype in thioredoxin-negative (TrxA$^-$) *E. coli* strains (Pille et al., 1990). In vivo studies of *R. sphaeroides* thioredoxin suggest that the enzyme can play a defensive role against oxidative stress (Pasternak, 1994).

The three-dimensional structure of *E. coli* thioredoxin has been studied by X-ray crystallography and NMR techniques (Holmgren et al., 1975; Katti et al., 1990; Jeng et al., 1994). The overall structure (Fig. 1 and Kinemage 1) consists of a β-pleated sheet flanked on either side by helices that delimit two clusters of conserved hydrophobic residues. One of these clusters (the "aliphatic" core) involves mostly aliphatic side chains: Ile 4, Leu 24, Ile 38, Ile 41, Leu 42, Ile 45, Ala 46, Leu 53, Val 55, Leu 78, Leu 80, Leu 99, Leu 103, and Leu 107. The second cluster (Fig. 1) includes a tetrahedral arrangement of four aromatic side chains (Phe 12, Phe 27, Phe 81, and Tyr 70) surrounded by aliphatic side chains. The redox-active dithiol-disulfide (Cys 32-Gly 33-

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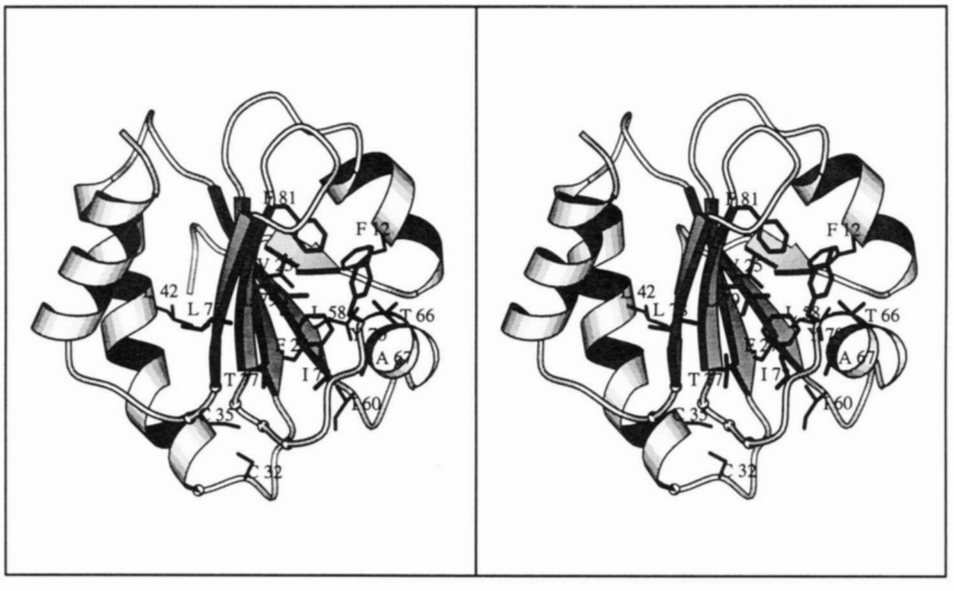


Fig. 1. Schematic stereo view of *E. coli* thioredoxin showing the hydrophobic residues discussed in the text. The active thiol/disulfide and the Cα positions of residues thought to be involved in protein–ligand interactions (white spheres) are indicated.

Pro 34-Cys 35) is found in a short loop that forms a protrusion on the molecular surface. This loop is probably implicated in protein–protein interactions with the viral gene 5 protein, the active subunit of DNA polymerase from phage T7 (Eklund et al., 1984).

The sequence homology between *E. coli* and *R. spheroides* thioredoxins strongly suggests a conserved overall structure with similar hydrophobic cores (Clément-Métral et al., 1988). In particular, both enzymes have the same number of aromatic residues because Tyr 70 of *E. coli* is replaced by a leucine residue, but a compensating mutation (Leu 79 → Phe) occurs in the same hydrophobic cluster of the *R. sphaeroides* enzyme. Other substitutions, however, tend to replace large aliphatic side chains in *E. coli* by smaller residues in *R. sphaeroides* (Leu 58 → Val, Ile 60 → Val, Ile 72 → Val), suggesting that a readjustment of the protein structure is required to preserve the tight packing of internal amino acids (Kaminsky & Richards, 1992).

Mutagenesis studies of thioredoxin have mostly involved conserved surface residues that could be important for catalysis or protein–protein interactions (reviewed by Eklund et al. [1991]). Relatively few studies, however, have investigated the effects of internal substitutions on thioredoxin stability and function. Langsetmo et al. (1990) showed that the mutation of Asp 26 to Ala increased the stability of the *E. coli* protein, suggesting that the buried aspartate could be critical for the enzyme function, whereas the mutation of Pro 76 (in a *cis* peptide bond) to Ala significantly decreased the stability of the protein (Gleason, 1992). Two amino acid residues at the center of the aliphatic core, Leu 42 and Leu 78 (Fig. 1), can be substituted by various side chains, including charged residues (Hellinga et al., 1992) and unnatural amino acids (Wynn & Richards, 1993) without significantly affecting the ability of the enzyme to support the in vivo assembly of filamentous bacteriophages. To further investigate the functional effects of internal substitutions in the hydrophobic core of thioredoxin, Phe 25 in the *R. sphaeroides* enzyme (corresponding to Phe 27 in *E. coli*) was replaced by amino acids with different side chain sizes and the resulting mutants were analyzed.

Results

The amino acid residue Phe 27 in *E. coli* thioredoxin occupies a central position within the hydrophobic cluster delimited by the β-sheet and helices α-1 and α-3 (Fig. 1 and Kinemage 1). This residue, largely conserved in homologous enzymes from different species, is not involved in the active site or in the protein region proposed to interact with the phage gene 5 protein. Five mutants of the equivalent residue in *R. sphaeroides* thioredoxin, Phe 25, were produced by replacing the phenylalanine amino acid for aliphatic or aromatic residues: Leu (F25L), Ile (F25I), Val (F25V), Ala (F25A), and Tyr (F25Y); and the modified proteins were characterized.

The ability of *E. coli* strains bearing the mutant *trxA* genes to grow on MetSO was analyzed to test for a functional enzyme (Table 1). At 37 °C, the strain expressing F25A thioredoxin showed low plating efficiency. In contrast, F25V, F25L, F25I, and F25Y mutants complemented the TrxA$^-$ phenotype with growth efficiencies between 44% and 60% that of wild type. The F25A mutant presented the same phenotype at 42 °C as at 37 °C and, although the protein was detected in crude extracts of *E. coli* by western blotting techniques, no colony was obtained at 30 °C (data not shown). Plating efficiencies for the other four mutants were equivalent to that for the wild-type protein, fully complementing the TrxA$^-$ phenotype at 42 °C. However, differences were observed in colony size. At 37 °C and at 42 °C, strains expressing F25V or F25Y thioredoxins appeared later and formed smaller colonies than strains expressing wild-type, F25L, or F25I thioredoxins. Because growth rates and homogeneous colony sizes were similar for all strains on medium supplemented with Met, the small size phenotype on MetSo is probably due to changes in the oxidoreductase activity of thioredoxin.

In a second experiment, thioredoxin mutants were tested for their ability to support growth of phage T3/7. At 37 °C, large

Table 1. *Biological functions of R. sphaeroides thioredoxin mutants*

Thioredoxins	Growth efficiency on MetSO[a,b]		Multiplication efficiency of T3/7 phage[a,c]		Multiplication efficiency of T7 phage[a,c]	
	37 °C	42 °C	37 °C	42 °C	37 °C	42 °C
Wild-type	1	1	1	1	1	1
F25A	$<8.10^{-7}$	ND	$<8.10^{-8}$	ND	0.26	$<8.10^{-8}$
F25V	0.44 (↓)[d]	1 (↓)	3.10^{-3}	6.10^{-5}	ND[e]	ND
F25L	0.615	1	$3.5.10^{-3}$	$<8.10^{-8}$	ND	ND
F25I	0.8	1	0.65	$<8.10^{-8}$	ND	ND
F25Y	0.77 (↓)	0.88 (↓)	3.10^{-3}	$<8.10^{-8}$	ND	ND

[a] Results obtained in the presence of 0.5 mM final IPTG.
[b] Expressed as the ratio of plaque numbers (mutants/wild type).
[c] Expressed as the ratio of percentage of colonies obtained on MetSO (mutants/wild type).
[d] ↓ indicates a smaller size of colonies on MetSO than those on Met.
[e] ND, not determined.

differences can be observed in multiplication efficiency (Table 1). F25A thioredoxin cannot support growth of phage T3/7 (even at 30 °C, data not shown). In contrast, it allows multiplication of the more virulent phage T7 with 26% efficiency that of the wild-type protein. Efficiency of plating of the phage T3/7 on F25V, F25L, and F25Y mutants was three orders of magnitude lower than that of the wild-type thioredoxin, whereas F25I can allow growth of phage T3/7 with native-like efficiency (65%). On the other hand, no mutant can support growth of phage T3/7 at 42 °C, indicating that the thioredoxin–gene 5 protein complex is thermosensible (Slaby & Holmgren, 1989).

Mutations equally affect plaque size and appearance. Plaques formed by phage T3/7 multiplication on strains expressing the *R. sphaeroides* wild-type thioredoxin are identical to those obtained by plating phages on the *E. coli* wild-type strain. However, infected mutant cells give indented plaques with heterogeneous sizes, these effects being more pronounced for the strain expressing F25V. Replication of phage T3/7 appears to be very sensitive to substitutions at position 25 because a significant functional difference is observed for two very similar mutants, F25L and F25I.

The capacity of thioredoxin mutants to serve as substrate for *R. sphaeroides* thioredoxin reductase was tested using the 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) assay. Catalytic efficiencies (Table 2) show that all mutant thioredoxins can act as substrate for thioredoxin reductase. Turnover rates (k_{cat}) for the reductase were greatly decreased when F25I or F25L was used as substrate. However, these changes are associated with a concomitant decrease of the apparent K_m, resulting in comparable catalytic efficiencies. The decrease in kinetic constants was less pronounced when F25V or F25Y was used as substrate, leading to slightly lower catalytic efficiencies. This result is consistent with the decrease of growth rate observed on MetSO.

Fluorescence emission intensity of tryptophans was measured as a function of increased concentrations of urea to analyze the stability of thioredoxin mutants. Fluorescence emission in *R. sphaeroides* thioredoxin, as in the *E. coli* enzyme, is primarily due to conserved tryptophan residues at positions 26 and 29 (28 and 31 in *E. coli*), immediately preceding the active site loop. The intensity of fluorescence for the oxidized form of the enzyme is significantly lower than that for the reduced or denatured forms, probably due to the quenching of fluorescence emission of Trp 26 by the active site (Holmgren, 1972; Kelley & Stellwagen, 1984). The emission spectrum of native oxidized *R. sphaeroides* thioredoxin has a maximum at 345 nm, which is shifted to 356 nm and increased threefold in the presence of 5 M guanidine·HCl (Clément-Métral, 1986). These fluorescence properties were similar for the wild-type and mutant thioredoxins in the presence of urea (instead of guanidine) as denaturant.

Denaturation curves of the proteins as a function of urea concentration are shown in Figure 2. (The quantity of purified F25A protein was not sufficient to carry out this study.) The urea midpoints for unfolding of all mutants are shifted to lower values, indicating that these proteins are less stable than the wild type. F25V displays the lowest urea midpoint (2.9 M), whereas those of F25L and F25I are slightly higher (4.7 M and 4.2 M, respectively). In contrast, the denaturation curve of F25Y is similar to that of wild-type thioredoxin.

The estimated free energy of stabilization of the *R. sphaeroides* wild-type protein, 8.2 kcal/mol, is similar to that of the *E. coli* enzyme, 8.7 kcal/mol (Kelley & Richards, 1987). The comparable stability of the two bacterial enzymes (despite several amino acid substitutions at internal positions) strengthens the plasticity of the protein backbone required to accommodate

Table 2. *Capacity of thioredoxin mutants to serve as substrates of R. sphaeroides thioredoxin reductase*[a]

Thioredoxins	K_m (μM)	k_{cat} (min^{-1})	Catalytic efficiency k_{cat}/K_m (min^{-1} μM^{-1})	Mutant/wild type
Wild type	3.27	1,228	332	1
F25V	1.5	300	200	0.6
F25L	0.42	150	357	~1
F25I	0.22	72	327	~1
F25Y	1.35	3,203	237	0.7

[a] Assays carried out at 23 °C in standard conditions as described in the Materials and methods. Reactions were initiated by addition of 10 nM *R. sphaeroides* thioredoxin reductase.

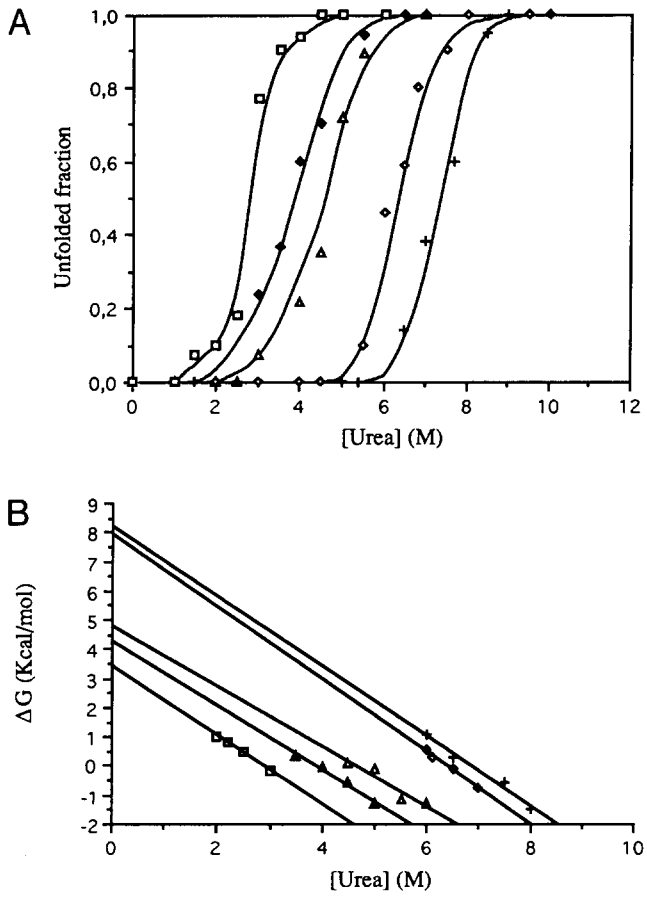


Fig. 2. Denaturation of mutant and wild-type thioredoxins in the presence of urea. **A:** Unfolded fractions as a function of urea concentration. **B:** Linear dependence between free energy and urea concentration. Symbols: +, wild type; □, F25V; △, F25L; ◆, F25I; ◇, F25Y.

these changes. On the other hand, all the mutants are less stable than the wild-type enzyme. In particular, the substitution of Phe 25 by aliphatic side chains decreases the stability of the protein by 3.4–4.8 kcal/mol.

Discussion

Previous studies revealed a high tolerance of the aliphatic hydrophobic core of *E. coli* thioredoxin to nonconservative substitutions, because virtually all studied variants were active in catalyzing the assembly of phage M13 (Hellinga et al., 1992). However, other functional effects of these substitutions, such as the oxidoreductase properties of the enzyme, were not investigated. In this paper we describe conservative substitutions of an internal hydrophobic residue that significantly affect both protein stability and biological function.

One of the mutants, F25A, has no detectable activity in the T3/7 phage test or in the DTNB assay, and the amount of recovered protein was 100-fold less than that for the wild type. This could imply a lower cellular concentration of the folded form of F25A. However, the low but significant activity obtained with the more virulent phage T7 suggests that the mutant retains the overall native conformation. Thus, as in the "extreme volume mutants" of λ repressor (Lim et al., 1992), the structure of thioredoxin may accept large modifications, but both stability and function are strongly affected.

The study of biological activities of the other four mutants expressed in the *E. coli* TrxA$^-$ strain reveals distinct functional effects, since thioredoxin variants with impaired capacity to support multiplication of phage T3/7 still retain oxidoreductase properties similar to those of the wild-type enzyme. Indeed, all the mutants are as active as wild-type thioredoxin in reducing MetSO. The comparable activities of the different mutants in reducing MetSO are further confirmed by the catalytic efficiencies measured in the DTNB assay. F25L and F25I proteins can serve as substrates for thioredoxin reductase as efficiently as the wild type. Some changes, however, are observed in the apparent K_m and k_{cat} values; the four mutants display better affinity for thioredoxin reductase and a lower rate of disulfide exchange. Since the location of Phe 25 in the three-dimensional structure precludes a direct role for this residue in catalysis (Fig. 1), the changes in kinetic parameters could be attributed to local structural modifications required to restore a closed packing of the protein interior. It is interesting to note that these results differ from those observed for the other mutations in the protein core of *E. coli* thioredoxin. For example, Langsetmo et al. (1990) showed that the mutual D26A of *E. coli* thioredoxin results in a very stable protein although, with a greatly reduced overall catalytic activity.

On the other hand, subtle disruptions of the protein interior significantly affect the capacity of the enzyme to support multiplication of phage T3/7. A quite distinct phenotype is observed for variants as similar as F25I (active) and F25L (inactive), or as the wild-type and the inactive F25Y. These results disagree with those obtained by Hellinga et al. (1992), for which major changes within the hydrophobic cluster failed to show functional differences in a similar assay. The two studies, however, are concerned with different regions of the protein interior. The apparent discrepancy could be explained, for example, if the interface between thioredoxin and the viral gene 5 protein extends beyond the active site region (Eklund et al., 1984) to include other parts of the molecular structure directly affected by substitutions at position 25.

Differences of stability between thioredoxin variants are correlated with the volume of substituent side chains but have no effect on the ability of the enzyme to reduce MetSO in vivo, nor can they explain the effects observed in phage assembly. For example, the F25L and F25Y mutants, unable to form an active phage complex, show stability comparable to active F25I and wild type, respectively. Thus, as previously suggested by Hellinga et al. (1992), thioredoxin may represent a class of proteins for which there is no evident correlation between biological activity and thermodynamic stability.

The present work demonstrates that conservative substitutions in the hydrophobic protein core can affect different biological functions in different ways. Similar observations have been previously reported by Krause and Holmgren (1991), who showed that substitutions of exposed Trp 31 within the active site region of *E. coli* thioredoxin strongly influenced the reduction of insulin disulfides or T7 DNA polymerase activity, but all the variants were good substrates for thioredoxin reductase.

Materials and methods

Oligonucleotides were synthesized by Eurogentec, Belgium. Isopropylthio-β-D-galactoside (IPTG), DTNB, nicotinamide ad-

enine dinucleotide phosphate reduced form (NADPH), bovin serum albumin (BSA), and urea, as well as antibiotics and amino acids, were obtained from Sigma Corp. [α-^{35}S]dCTP was from Amersham Corp. (England). Restriction endonucleases and DNA-modifying enzymes were obtained from Boehringer GmbH (Mannheim, Germany) or Eurogentec. *E. coli* thioredoxin was from IMCO Corp. Ltd. AB (Stockholm, Sweden). Antibody anti-sheep IgG peroxidase conjugate was obtained from Sigma chemical. *R. sphaeroides* thioredoxin reductase was from homogeneous preparation available in this laboratory (Clément-Métral, 1986).

Bacterial strains, phages, and plasmids

E. coli strains W3110, A523 (W3110ΔtrxA), A312 (K38*metE163*::Tn10) (Russel & Model, 1986) and bacteriophage T3/7 (Huber et al., 1986) were a generous gift from Dr. Marjorie Russel (Rockfeller University, New York). Pr. A. Holmgren (Karolinska Institut, Stockholm) kindly supplied bacteriophage T7. JAS46 (A523 *metE163*::Tn10) was constructed by transduction of *metE163*::Tn10 from A312 to A523. *E. coli* Xl1Blue (*recA*, *endA*, *gyrA96*, *thi*, *hsdR17*, (rk^-, mk^-), *supE44*, *relA1*, lac[F′, *proAB*, *lacI*^{q}, ZΔM15, Tn10]) (Bullock et al., 1987) obtained from Stratagene (La Jolla, California), was used for phage M13 multiplication. Expression vector pUTC51, which contains the f1 intergenic region and the *lacI*^{q} gene encoding the lac repressor, was derived from pKK233-2 (Amann and Brosius, 1985). The *trxA* gene encoding *R. sphaeroides* thioredoxin is cloned under the control of the strong *trc*-promoter. The *E. coli* $TrxA^-$ mutant containing pUTC51 has the same phenotype, in presence of IPTG, as the W3110 *E. coli* wild-type strain.

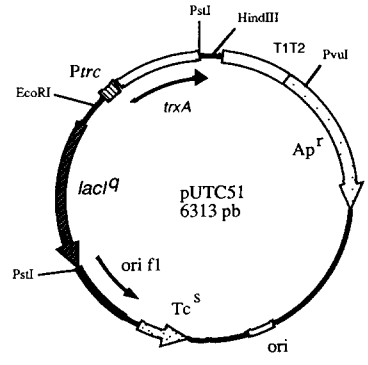
DNA cloning techniques

Enzymatic modifications, electrophoresis, and elution were carried out according to Sambrook et al. (1989). The kit FlexiPrep (Pharmacia LKB) was used for double-stranded DNA extractions. DNA sequencing was carried out on the denatured double-stranded plasmid or on the single-stranded phage DNA (T^7 sequencing kit, Pharmacia LKB). Preparation of single-stranded DNA from phage M13 was performed as described by Veira and Messing (1987). Cell transformation and transfection were carried out as described by Hanahan (1985).

Site-directed mutagenesis

Thioreoxin mutants were constructed by oligonucleotide-directed site-specific mutagenesis on the single-stranded *trxA* gene subcloned in the M13mp18 vector. The *Eco*R I-*Hin*d III fragment containing the *R. sphaeroides* gene *trxA* was isolated from pUTC51 expression vector (Fig. 3) and subcloned within the polylinker region of the M13mp18 vector. After XL1Blue transfection, plaques obtained on agar plates were used to infect a culture of XL1Blue strain to produce phage particles. The single-stranded DNA was extracted to serve as template for mutagenesis. "Oligonucleotide-directed in vitro mutagenesis" kit (Amersham) was used to construct the five mutants following the instructions of the supplier. The screening was directly made by sequencing on the single-stranded DNA with specific oligonucleotide primers. The *trxA* gene was then entirely sequenced to verify that no other mutations were introduced. The yield obtained for each mutant was higher than 50%.

A



B

F25A:	5'CA TTC GGC CCA GGC ATC GAC GAC GAC3'
F25V:	5'CA TTC GGC CCA GAG ATC GAC GA3'
F25I:	5'CA TTC GGC CCA GAT ATC GAC GA3'
F25L:	5'CA TTC GGC CCA AAC ATC GAC GA3'
F25Y:	5'CA TTC GGC CCA GTA ATC GAC GA3'

Fig. 3. Expression and site-directed mutagenesis of the *trxA* gene. **A:** Physical map of pUTC51 expression vector. *Eco*R I-*Hin*d III, containing the *trxA* gene, was subcloned within the polylinker region of the M13mp18 vector to construct mutant *trxA* genes. The *lacI*^{q} gene encodes for the *lac* repressor. **B:** Mutagenesis primers used to substitute F25 amino acid by site-directed mutagenesis.

The phage particles containing the mutant *trxA* genes were used to transfect the XL1Blue strain in liquid medium. The *Eco*R I-*Hin*d III fragments were then isolated and ligated to the pUTC51 vector after elimination of the wild-type *trxA* gene. The presence of the mutation was confirmed by sequencing with specific oligonucleotide primers. The recombinant vectors were used to transform the host strain JAS46 lacking the *trxA* gene.

Protein expression and purification

Transformed strains were grown in 1.5 L medium Haiech (Laboratoire de Chimie Bacterienne, CNRS, Marseille), containing 200 μg/mL ampicillin to the start of the exponential phase. IPTG (0.5 mM) was added to the culture at the beginning of the exponential phase. The culture was then continued overnight at 37 °C with a vigorous aeration. Cells were harvested during the stationary phase and stored frozen at −20 °C.

Frozen cells were taken up in 4 times their weight of 10 mM potassium phosphate buffer, pH 7.4, 1 mM EDTA and disrupted by passing through a french pressure cell. Crude extracts were prepared by centrifugation for 3 h to 250,000 × *g*. Nucleic acids were precipitated by addition of 20% streptomycin sulfate to a final concentration of 1%. For wild-type thioredoxin, the supernatant was heated for 10 min at 80 °C, rapidly cooled, and centrifuged to eliminate the precipitate. Supernatant solutions were brought to 80% saturation with solid ammonium sulfate, the pellet resuspended in minimum volume of 0.5% NH_4HCO_3, and dialyzed against the same buffer. Fractions containing thioredoxin were detected by western blotting (mutant) or DTNB activity (wild type), and were pooled and concentrated by lyophilization. The purity of thioredoxins was checked by Coomassie blue staining on SDS-PAGE.

Analytical methods

Protein concentration was determined from absorbance at 280 mm using molar extinction coefficients of 13,700 M^{-1} cm^{-1} for wild-type and mutant thioredoxins F25A, F25V, F25L, F25I, and 15,000 M^{-1} cm^{-1} for mutant thioredoxin F25Y. For thioredoxin reductase, a molar extinction coefficient of 10^5 M^{-1} cm^{-1} was used (Clement-Métral, 1986). Native and SDS page were carried out as described by Schägger and von Jaggow (1987). For western blotting, proteins were transferred to nitrocellulose by electrotransfer. The membrane was blocked for 1 h at 37 °C in 3% BSA, washed, and incubated with 1:600 diluted anti-*E. coli* thioredoxin in 1% BSA/Tween 0.1% for 3 h at 37 °C. Excess of antibody was washed out and anti-rabbit IgG alkaline phosphatase conjugate was added to 1:2,500. The membrane was incubated, washed, and, after 1 h at 37 °C, the reactive bands were detected by alkaline phosphatase conjugate.

Denaturation curves

Protein unfolding was monitored by following the tryptophan fluorescence as a function of denaturant concentration on a Perkin-Elmer spectrofluorimeter LS250. Measurements were made in a 1.5-mm-pathlength cell (LC cell) with a total volume of 100 μL. Fluorescence was excited at 280 nm and recorded between 300 nm and 400 nm. The samples contained about 5 μM of thioredoxin and different concentrations of urea as denaturant, in 20 mM potassium phosphate, pH 7.4. They were allowed to stand at 23 °C for 30 minutes before fluorescence was recorded. Relative intensity changes were determined under the fluorescence spectra from 300 nm to 400 nm. Unfolding curves were analyzed as described by Pace (1980).

Enzymatic assays

The ability of thioredoxins to serve as substrates for *R. sphaeroides* thioredoxin reductase was analyzed by monitoring DTNB reduction in the presence of NADPH (Slaby & Holmgren, 1979). Assay mixtures contained 100 mM Tris-HCl, pH 7.8, 2 mM EDTA, 0.1 mg/ml BSA, 0.5 mM DTNB, and 0.24 mM NADPH in 500 μL final volume. Reactions containing thioredoxins in the range from 0.05 to 10 μM were started by addition of 10 nM *R. sphaeroides* TR. The reduction of DTNB was followed at 412 nm, ϵ_{412}(DTNB) = 13,600 M^{-1} cm^{-1}, in a 1-cm-pathlength cell at 23 °C on a Cary 1 spectrophotometer.

Assays of thioredoxin function in vivo

The various strains expressing wild-type and mutant thioredoxins were plated on the M9 minimal agar described by Sambrook et al. (1989) complemented with 50 μg/mL Met (methionine) or 50 μg/mL MetSO (methionine sulfoxide). When required, IPTG was added at 0.5 mM final concentration. *E. coli* strains W3110 and JAS46 were grown as positive and negative controls. After incubation at 37 °C or 42 °C overnight, plating efficiencies were determined by the ratio of colony number on minimal medium containing MetSO to that containing Met.

Test strains for the MetSO reduction assay were also used to evaluate the viability of pages T7 and T3/7. Strains were grown at an Abs_{580} = 0.5 and infected with phage lysates at the appropriate dilution. The infected cells were plated on agar plates incubated overnight at 37 °C or 42 °C.

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